Universidade de Aveiro Departamento de Biologia

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Desenvolvimento de modelos de células de mamífero para estudo da AIP56

Development of a mammalian cell model to study AIP56

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada no ramo de Biologia Molecular e Celular, realizada sob a orientação científica da Professora Doutora Ana Maria Silva do Vale, Investigadora Auxiliar no Instituto de Biologia Molecular e Celular no Porto e co-orientação científica do Professor Doutor Carlos Miguel Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

“You must learn to question everything. To wait before moving, to look before stepping, and to observe everything”

Nicholas Flamel

o júri

presidente

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agradecimentos

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palavras-chave

Transfecção, *Photobacterium damselae* subsp. *piscicida*, AIP56, toxinas AB, apoptose, NF-kB

resumo

A photobacteriose de peixes é uma infecção sistémica de evolução rápida, causada pela bactéria Gram-negativa *Photobacterium damselae* subsp. *piscicida* (*Phdp*), que provoca elevadas mortalidades em várias espécies de peixes marinhos. Nos animais infectados, a *Phdp* dissemina-se na corrente sanguínea e secreta a AIP56, uma exotoxina apoptogénica que permite ao agente infeccioso escapar à defesa fagocítica do hospedeiro através da indução da morte por apoptose dos fagócitos. Embora tenha sido demonstrado que a AIP56 é um factor-chave da virulência da *Phdp* e embora a patologia associada à AIP56 esteja bem caracterizada, os alvos moleculares da toxina e as vias moleculares por ela afectadas continuam por desvendar. Dados recentes revelaram que a AIP56 é uma toxina do tipo AB, contendo um domínio N-terminal (domínio A) de metaloprotease responsável pela actividade catalítica e um domínio C-terminal (domínio B) envolvido na ligação e internalização da toxina pelas células. O domínio N-terminal da AIP56 é homólogo ao *non-LEE encoded effector C* (NleC), um efector presente em várias bactérias patogénicas entéricas e secretado por um sistema de secreção do tipo III. Foi recentemente descrito que o NleC corta e inactiva o p-65 do NF-kB. Contudo, continua por investigar se a AIP56 também tem como alvo o p-65 do NF-kB e se existe uma relação entre um possível corte do p65 e consequente inactivação do NF-kB e a actividade apoptogénica da toxina. Embora se tenha avaliado a susceptibilidade de várias linhas de celulares de mamíferos à AIP56, pelo facto de não se ter observado a ocorrência de apoptose como consequência da exposição à toxina em nenhuma das linhas testadas, até ao momento, a actividade da AIP56 tem vindo a ser estudada usando um modelo *ex vivo* que consiste em leucócitos peritoneais isolados de robalo. A especificidade observada poderá resultar do facto de os receptores de membrana para a AIP56 existirem nos fagócitos dos hospedeiros susceptíveis e estarem ausentes nas células de mamífero, sugerindo que se a AIP56 entrasse nas células de mamífero, poderia ser capaz de induzir apoptose dessas células. A existência de um modelo celular de mamífero para estudar os detalhes da actividade da AIP56 seria muito vantajosa, pois permitiria utilizar um vasto leque de ferramentas disponíveis comercialmente que não existem ou não podem ser usadas em estudos com células de robalo. Neste trabalho testamos e optimizamos diferentes protocolos para introduzir a AIP56 e proteínas derivadas da AIP56 em células HeLa. Dos resultados obtidos, conclui-se que os métodos mais eficazes para a introdução da toxina foram a transfecção por método químico e a utilização do sistema LF/PA do *Bacillus anthracis*. A transfecção química permite não só obter e estudar células transfectadas de forma transiente, mas também desenvolver linhas celulares transfectadas estáveis.

keywords

Transfection, *Photobacterium damselae* subsp. *piscicida*, AIP56, AB-toxins, apoptosis, NF-kB

abstract

Fish photobacteriose is a bacterial systemic and deadly infection with rapid course and very high mortalities, caused by the *Photobacterium damselae* subsp. *piscicida* (*Phdp*). *Phdp* spreads through the bloodstream and secretes the apoptogenic exotoxin AIP56 allowing the pathogen to avoid phagocytosis by inducing the apoptotic death of the host phagocytes. Although AIP56 was found to be a key virulence factor of *Phdp* and the AIP56-related pathology has been well characterized, the molecular targets of the toxin and the molecular pathways it affects/modulates remain to be disclosed. Recent data revealed that AIP56 is an AB-toxin, possessing an N-terminal metalloprotease domain (A domain) responsible for the catalytic activity of the toxin and a C-terminal domain (B domain) involved in the binding/internalization of the toxin into the cells. The N-terminal domain of AIP56 is homologous to the non-LEE encoded effector C (NleC), a type III secreted effector of enteric pathogenic bacteria that cleaves and inactivates the p65 of NF-kB. However, it remains to be investigated if AIP56 also targets NF-kB p65 and if NF-kB p65 inactivation by AIP56 is linked to the apoptogenic activity of the toxin. So far, AIP56 activity has been being studied using an *ex vivo* model consisting of freshly isolated sea bass peritoneal leukocytes because although the susceptibility of several mammalian cell lines to AIP56 has been assessed, no apoptosis was observed in any of the cell lines tested. This specificity of the toxin may result from the existence of receptors for AIP56 in phagocytes of susceptible hosts and their absence in mammalian cells and suggests that if AIP56 was able to enter mammalian cells, it would be able to induce apoptosis in those cells. The existence of a mammalian cell model for studying in detail the AIP56 activity would be very advantageous, because there is much more availability of tools to study mammalian cells than sea bass cells. In this work, we tested and optimized different protocols for the intracellular delivery of AIP56 and AIP56-related proteins into HeLa cells. We found that the most efficient methods for intracellular toxin delivery were chemical transfection with toxin-encoding expression vectors and the use of the LF/PA system of *Bacillus anthracis*. The chemical transfection allows not only to obtain and study transiently transfected cells, but also to develop stable transfected cell lines.

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Introduction

Fish photobacteriosis

Fish photobacteriosis is a bacterial septicemia caused by *Photobacterium damselae* subsp. *piscicida* (*Phdp*). This Gram-negative bacterium is highly pathogenic for wild and cultured marine fish, affecting more than 20 fish species worldwide. In the acute form, photobacteriosis is a systemic and deadly infection with rapid course and very high mortalities [1, 2].

Fish photobacteriosis was first reported in wild populations of white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) in 1963 in Chesapeake Bay, USA. In 1969, the pathogen has become economically important, as it started to cause losses of fish in Japan, compromising the Japanese fish farming industry [3]. In 1990, many outbreaks of photobacteriosis happened in cultured sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) in different European countries [1]. Although epizootic can be controlled by treatment with antibiotics and some aquacultures apply immunoprophylaxis in broodstock and juveniles, presently, fish photobacteriosis continues to be a serious problem in the intensive culture of different fish species in the Mediterranean area and Japan, causing considerable economic losses in the marine aquaculture industry in these regions [3, 4].

External pathological signs of fish photobacteriosis are not immediately obvious, as surface lesions are usually absent in infected fish. However, a dark coloration and a decrease in natatorium activity may occur. Internally, infected fish exhibit haemorrhagic septicemia, and granulomatous lesions in the kidneys, spleen and liver due to the accumulation of bacteria [5]. It has been shown that in infected fish, *Phdp* spreads through the bloodstream, colonizes various internal organs and secretes the apoptogenic exotoxin AIP56 (apoptogenic inducing protein of 56 kDa), that allows the pathogen to avoid phagocytosis by inducing the apoptotic death of the host phagocytes [2]. Phagocytosis is the first crucial step of the antibacterial host defence mechanism against extracellular pathogens. Many of these pathogens are able to escape from phagocytosis through the use of molecules that efficiently kill host phagocytes. A mechanism used by extracellular pathogens to successfully escape phagocytosis is the production of molecules that

destroy the phagocytes of the host, usually macrophages and neutrophils, by apoptosis.

Apoptosis is an evolutionarily conserved process used by multicellular organisms to developmentally regulate cell number or to eliminate cells that are potentially detrimental to the organism, as an instrument for the disposal of unwanted cells during embryonic development, during normal cell turnover in proliferating tissues and in pathological situations, maintaining the homeostasis [6]. A cell undergoing apoptosis loses the connections with other cells and the extracellular matrix and rounds up. It begins to display the protrusions of the plasma membrane, usually referred to as ‘blebs’. Blebs can be seen by some time protruding and retracting. Condensation of the cell nucleus occurs, often starting as a condensed ring along the nuclear envelope, and then extends to encompass the entire nucleus. The condensed nucleus may disassemble into multiple fragments. The cell condenses and is reorganized into so-called ‘apoptotic bodies’. Caspases are activated and their activity is required for the observed changes in morphology [6-10].

Under normal conditions, apoptotic cells and apoptotic bodies are eliminated by removal via scavengers engaged by the exposure of engulfment signals during the implementation phase of apoptosis [11]. This is a mechanism for eliminating cells with the participation of a scavenger cell to engulf and digest by heterolysis the apoptosing cell before post-apoptotic secondary necrosis occurs, that is, while the cell in apoptosis is still surrounded by a nearly intact cytoplasmic membrane [7]. Phagocytosis of apoptosing cells is carried out mainly by macrophages [11]. When the removal of the apoptotic cells and apoptotic bodies by scavengers fails, the apoptotic process transits to secondary necrosis [12]. Secondary necrosis involves the activation of self-hydrolytic enzymes, swelling of the cell or apoptotic bodies and irrecoverable damage to the cytoplasmic membrane, leading to the disassembling of the cell with release of the cellular components [11, 13].

As already mentioned, AIP56 is the protagonist of the phagocytic elimination that occurs in photobacteriosis. The toxin activates a mechanism of endogenous programmed cell death of the host macrophages and neutrophils,

leading to their elimination by apoptosis [14]. One of the consequences of that elimination is a lack of functional phagocytes that leads to an insufficient clearance of apoptotic cells and apoptotic bodies that eventually lyse by secondary necrosis [2, 15]. The phagocyte apoptosis induced by AIP56 proves to be a very effective mechanism of amplification of the etiopathogenicity, because it results in two effects that act together against the host: evasion of the pathogen from phagocytosis, a fundamental defence mechanism, and release of tissue harmful molecules by secondary necrosis of the apoptotic phagocytes [2, 15].

AIP56 and NleC

Analysis of the AIP56's primary structure shows that it has an N-terminal cleavable signal peptide of 23 amino acid residues (Figure 1), that is cleaved during secretion, originating a mature protein with 497 amino acid residues [14, 15].

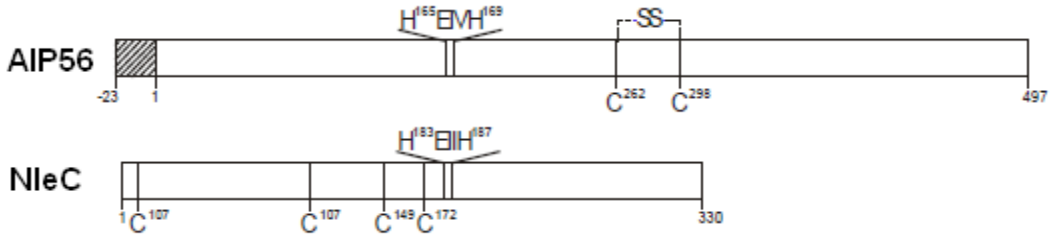


Figure 1. Schematic representation of AIP56 and NleC. Slashed box represents the AIP56's signal peptide. The conserved zinc-metalloprotease signature (HExxH) and the cysteine residues are represented and numbered at their relative positions. The disulfide bridge between cysteine 262 and 298 of AIP56 is depicted. Adapted from [15].

Homology analysis showed that the N-terminal region of AIP56 (first 324 amino acids) is homologous to the non-LEE (locus of enterocyte effacement) encoded type III secreted effector C (NleC) conserved in many pathogenic enteric bacteria (like enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella enterica* and *Citrobacter rodentium*) while the C-terminal of AIP56 is homologous to an unknown protein of the temperate lambda-like bacteriophage APSE-2, suggesting that AIP56 is a two-domain protein. The two-domain structure of AIP56 was confirmed by limited proteolysis of the toxin with chymotrypsin. Controlled digestion of AIP56 with chymotrypsin originates two fragments, resulting from cleavage between F^{285} and F^{286} [15].

These fragments are consistent with the domains predicted by homology analysis and were found to remain linked by a disulfide bridge between C^{268} and C^{298} (Figure 1) [15].

An important feature of AIP56 is the presence of the zinc-binding signature HExxH that is typical of most zinc metallopeptidases (Figure 1). This signature is present in the AIP56 N-terminal domain and is conserved in the NleC. This information led to the hypothesis that AIP56 is an AB-toxin, having an A domain (N-terminal domain) exhibiting metalloprotease activity connected to a B domain (C-terminal domain) by a disulfide bridge [15]. AB–toxins are so named because they consist of two parts, an A component that is the active catalytic domain and a B component that binds to the surface of the host cell membrane, allowing the entry of the A domain into the cell. Most AB-toxins are endocytosed, and translocate from the endosomal compartment or endoplasmic reticulum to the cytosol, while others bind to the host cell and the A component subsequently passes directly through the host cell's membrane and enters the cytoplasm [16].

NleC is a type-III secreted zinc metalloprotease targeting the transcription factor NF-kB [17-20], which function is to regulate the inflammatory and apoptotic response [21, 22] (Figure 2). The homo- or heterodimeric NF-kB complexes are composed by two of five related proteins, among them p-65. Normally, NF-kB dimers are retained in the cytoplasm by association with inhibitory proteins termed IkBs. Due to different stimuli, the IkBs are phosphorylated by IkBs kinases (IKK). This triggers IkB ubiquitination, leading to its proteasome-dependent degradation and consequently allowing translocation of the free NF-kBs to the nucleus [23, 24]. NF-kB is the main signalling pathway employed by the host cells to respond to microbial threats [25]. The most important activators of the microbial-induced NF-kB are the pattern recognition receptors (PRRs), a set of transmembrane or intracytoplasmic receptors that specifically recognize and bind different microbial macromolecular ligands named microbial-associated molecular patterns (MAMPs), such as lipopolysaccharide (LPS), flagelin, peptidoglycans and microbial nucleic acids [25]. The cytokines and MAMPs activate NF-kB, by engagement of their cognate cells' surface receptors. In the nucleus, NF-kBs in their active forms, bind to the DNA and activate expression of genes that mediate release of antimicrobial

molecules, cytokines, chemokines, adhesion molecules or receptors involved in immune recognition process [25]. The inflammatory cascade begins when recognition of MAMPs by local cells results in NF-kB activation and consequent release of cytokines that promote macrophage re-localization and activation at the site of infection. Together, antimicrobial molecules and recruited macrophages cooperate to kill pathogens, clear infection, and remove dead cells [25].

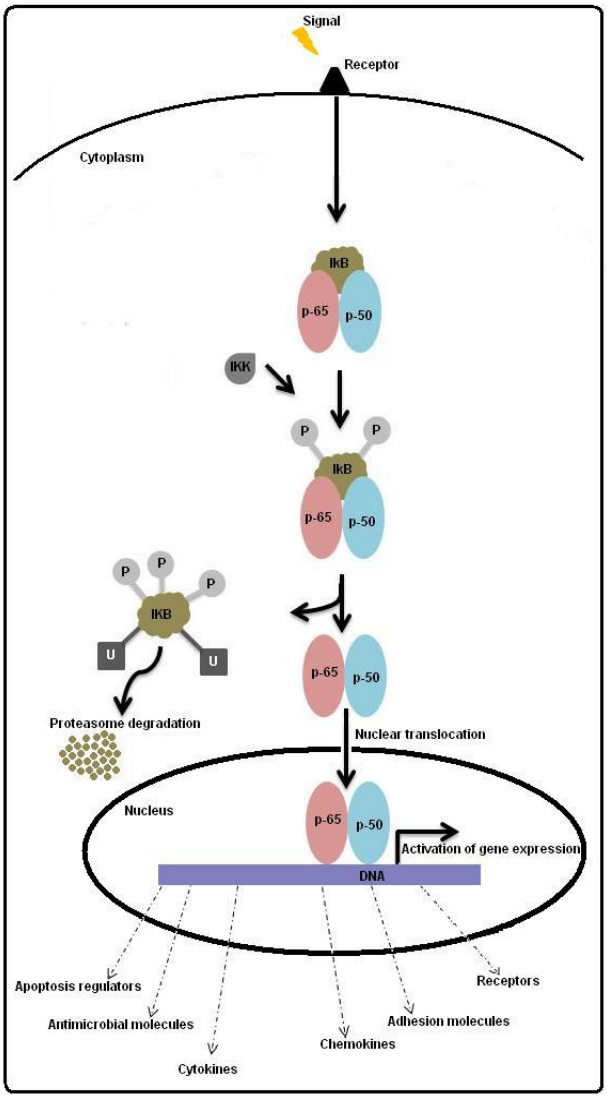


Figure 2. Schematic representation of the NF-kB pathway. Stimulus activation of IKK leads to the phosphorylation and consequent ubiquitination of IkB, that enters into proteasome degradation and frees the NF-kB, which translocates to the nucleus, bind the DNA and activate the expression of genes involved in the inflammatory and anti-apoptotic responses.

In addition to activating the expression of genes involved in the control of the immune and inflammatory response, the NF-κB pathway is also a key mediator of genes involved in the inhibition of apoptosis, including the cellular inhibitors of

apoptosis (c-IAP1, c-IAP2, and XIAP), the tumor necrosis factor (TNF) receptor-associated factors (TRAF1 and TRAF2), the Bcl-2 homologues A1/Bfl-1, Bcl-XL and IEX-1L [22, 26, 27]. The antiapoptotic proteins block the activation of caspases involved in the apoptotic pathway [27], induced by TNF and other stimuli [28].

NleC has been found to inhibit p-65 through its cleavage at the N-terminus. The inactivation of the p-65 subunit of NF-kB is followed by proteasomal degradation, leading to the loss of its transcriptional activity, and to the failure of the immunological process in the cells, resulting in their inability to fight infection [17-20, 29].

Although no substrates for AIP56 were identified when the present work started, the above described homology between the N-terminal domain of AIP56 and the NleC suggested that both proteins could have a common substrate, that is, that AIP56 also targets and inactivates NF-kB p-65, compromising the anti-apoptotic response of the affected host cells and leading to their elimination by apoptosis.

Objectives

The biological activity of AIP56 has been studied using primary cultures of fish leukocytes, because there are no macrophage cell lines from susceptible fish and it has been found that AIP56 does not induces apoptosis in mammalian cell lines. This specificity could be due to the existence of receptors for AIP56 in phagocytes of susceptible hosts and their absence in mammalian cells. Therefore, we hypothesized that if AIP56 was able to enter mammalian cells, it would be able to induce apoptosis in those cells. A mammalian cell model for studying AIP56 activity would be very advantageous, because there are more tools (antibodies and subcellular markers, for example) available to study mammalian cells than sea bass cells.

This study aimed at developing a mammalian cell model to study AIP56 activity. To achieve this goal, the following strategies were followed:

1. Intracellular delivery of AIP56-related proteins by transfection and establishment of stable-transfected cell lines.
2. Intracellular delivery of AIP56 subunits using the LF-PA system of *Bacillus anthracis*

Material and Methods

In vitro cleavage of NF-kB p-65 by AIP56

Cell culture

HeLa Tet-On cells (Clontech) were maintained in Dulbecco's Eagle's Medium (DMEM; Gibco), with 10 % Fetal Bovine Serum Tetracycline Free (FBS Tet-Free; Clontech), 1% G418 (Gibco) and 1% Penicillin-Streptomycin (PenStrep; Lonza), at 37ºC in humid atmosphere with 5% CO_2. Cells were subcultured whenever approximately 75% confluence was reached.

Preparation of cell lysates and in vitro cleavage assays

The cells were lysed with lysis buffer (10 mM Tris-HCl pH 8.0, 0.5% Triton-X-100, 150 mM NaCl, 10% glycerol), with the proportion of 20 µl per $1.0x10^6$ cells and centrifuged at 16100 x g, for 15 minutes (min) at 4ºC, and the soluble fractions were transferred to new eppendorf tubes. The cleavage of NF-kB p-65 was assessed by incubation of 20 µl of HeLa Tet-On cells soluble faction with 0.1 µM of AIP56 or AIP56-derived proteins in the presence or absence of 5 mM of the metalloprotease inhibitor of 1,10-phenanthroline (Phe), for 3 hours (h) at 22ºC. The following proteins were tested: full-length AIP56 (AIP56), a mutated version of AIP56 carrying amino-acid substitutions that disrupt the zinc metalloprotease signature (AIP56mut), an AIP56 N-terminal truncate corresponding to the putative AIP56 A domain (AIP56N) and an AIP56 C-terminal truncate corresponding to the putative AIP56 B domain (AIP56C). Recombinant NleC was used as a positive control. All the proteins were produced and characterized at the Fish Immunology and Vaccinology group of IBMC and have a His-tag at the C-terminus.

SDS-PAGE and western-blotting

The cell lysates were mixed with Gel Loading Buffer (GLB; 50 mM Tris-HCl, pH 8.8, 2% SDS, 10% glycerol, 0.017% bromophenol blue, 2 mM EDTA pH 8.8, 100 mM DTT) and denatured by heating at 95ºC for 5 min. The proteins were resolved in 10% SDS-polyacrylamide gels (Figure 3).

Components	Running Gel (10%)	Stacking Gel
30% acrylamide / 0.4% Bisacrylamide	5 ml	830 µl
1.875 M Tris-HCl pH 8.8	6 ml	-
0.6 M Tris-HCl pH 6.8	-	1.05 ml
H_2O	3.84 ml	3.02 ml
SDS 10%	150.40 ml	50 µl
TEMED	10.80 µl	3 µl
10% APS	100.80 µl	25 µl

Figure 3. Composition of the running and stacking gels

The gels were mounted in an electrophoresis apparatus and electrophoresis buffer (25 mM Tris-Base, 250 mM glycine, 0.1% SDS) was added to the top and bottom reservoirs. Samples were loaded into the wells and the gels were run at 80 V.

Proteins in the gels were transferred to nitrocellulose membranes in transfer buffer (25 mM Tris-Base, 192 mM glycine, 20% methanol), for 1 h at 21 V. To control transfer efficiency, equal loading of the lanes, and to locate the molecular weight markers, the membranes were stained with Ponceau S (Sigma). The membranes were blocked for 1 h at room temperature (RT) in tris buffered saline (TBS; 20 mM Tris-, 137 mM NaCl, pH 7.6) with 0.1% Tween 20 (Sigma; T-TBS) and 5% low fat powdered milk. Rabbit antibody anti-c-terminal region of human NF-kB p-65 (C20; Santa Cruz-Biotechnology), diluted in T-TBS (1:7500), was added to the membrane and incubated with shaking for 1 h at RT. The membranes were washed 3 times for 5 min in T-TBS, at RT with shaking, and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (The Binding Site), diluted in T-TBS (1:30000) for 1 h at RT. After 3 washes for 5 min in T-TBS, at RT with shaking, the reactive bands were detected using enhanced chemiluminescence substrate (ECL; SuperSignal® West Pica, Thermo Scientific) following the manufacturer's instructions.

Intracellular delivery of AIP56-related proteins by transfection

To delivery of AIP56 by transfection, we decided to use HeLa Tet-On cells that allow us to regulate gene expression adding doxycycline (Dox) to the culture medium. The Tet-On system for controlling expression of genes of interest in mammalian cells was developed by Hermann Bujard and Manfred Gossen at the University of Heidelberg [30] as shown in Figure 4. This system enables to tightly and reversibly control expression of proteins with the addition of Dox and is useful in *in vivo* studies when rapid activation of a gene is required.

The Tet regulatory system is performed by the Tet repressor protein (TetR) that negatively regulates the genes of the tetracycline-resistance. TetR blocks transcription of these genes by binding to the tet operator sequences (tetO) in the absence of Dox. TetR and tetO provide the basis of regulation and induction for use in mammalian experimental systems [30]. A “reverse” Tet repressor (rTetR) was created by four amino acid changes in TetR, and the combination with the C-terminal domain of VP16 from Herpes Simplex Virus (HSV), converted the rTetR into the reverse tetracycline-controlled transactivator (rtTA), an hybrid protein that activates transcription in the presence of Dox [31].

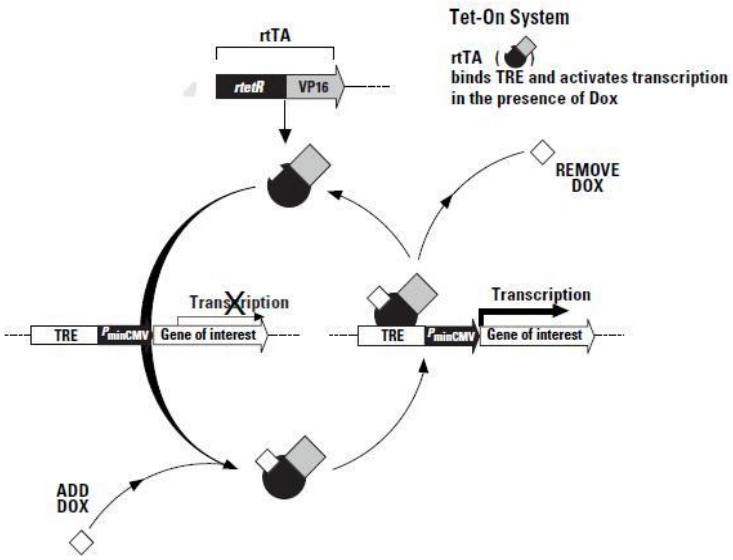


Figure 4. Schematic representation of the regulation of gene expression in response to Dox in a Tet-On system.

The rtTA binds the tetracycline-response element (TRE) that is present in the response plasmid, which expresses a gene of interest. The TRE consists of seven direct repeats of a 42-bp sequence containing the tetO, and is located just upstream of the minimal cytomegalovirus (CMV) promoter (P_{minCMV}). In this system, high levels of the gene of interest are expressed in the presence of the system's inducer, the Dox.

Transient transfection

Cell culture

HeLa Tet-On cells were maintained in DMEM with 10% FBS Tet-Free at 37ºC in humid atmosphere with 5% CO_2. Cells were subcultured whenever approximately 75% confluence was reached.

Plasmid purification

Vectors for transfection were constructed in the pTRE2hyg2-Myc vector at the Fish Immunology and Vaccinology group of the IBMC, as shown in Figure 5. They express a hygromycin (Hyg) resistance gene, permitting easy selection of stable transfectants and also encode a c-Myc tag, which is incorporated at the N-terminus of the expressed proteins to screen directly for protein expression by western-blotting and immunofluorescence (IF). A Tet-responsive promoter (P_{hCMV-1}) is also present in the vectors [30-34].

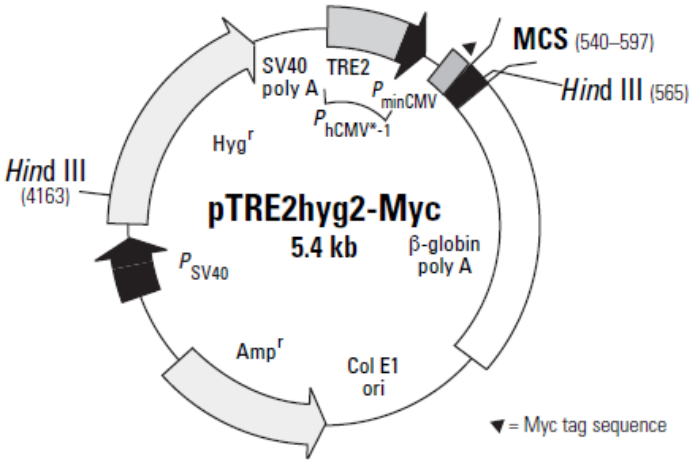


Figure 5. Schematic representation of the pTRE2hyg2-Myc vector

In this work, the following plasmids were used:

- pTRE2hyg2-Myc-AIP56: coding for c-Myc tagged full length AIP56 (AIP56).
- pTRE2hyg2-Myc-AIP56mut^{AAIVA}: coding for c-Myc-tagged mutated version of AIP56 (AIP56mut) carrying amino-acid substitutions that disrupt the zinc metalloprotease signature (HEXXH). The mutations consist on the substitution by alanines of the key residues for zinc ion coordination and water molecule activation: His165, Glu166, His169 and His170.
- pTRE2hyg2-Myc-AIP56N: coding for c-Myc-tagged AIP56$^{1-285}$ (Asn^{1} to Phe^{285}; AIP56N) corresponding to the putative AIP56 A domain.
- pTRE2hyg2-Myc-AIP56Nmut$^{AAIVA:}$ coding for c-Myc-tagged mutated version of putative AIP56 A domain (AIP56Nmut).
- pTRE2hyg2-Myc-AIP56C: coding for c-Myc-tagged AIP56$^{286-497}$ (Phe^{286} to Asn^{497}; AIP56C) corresponding to the putative AIP56 B domain.
- pTRE2hyg2-Myc-NleC: plasmid coding for c-Myc-tagged non-locus of enterocyte effacement encoded type III secreted effector C (NleC).

Escherichia coli XLI were transformed with the different plasmids, by the heat and shock method. Transformants were plated in luria bertani (LB)-agar plates containing 150 µg/ml ampicillin and incubated overnight (ON) at 37ºC. On the next day, starter cultures were made from single colonies that were picked and inoculated in LB medium containing ampicillin and incubated approximately 8 h at 37°C with shaking (170 rpm). The starter cultures were used to inoculate 500 ml of fresh medium containing ampicillin. The culture was were grown at 37°C ON, with shaking (170 rpm), and the plasmids were purified according to the protocol of the commercial QIAGEN® Plasmid Maxi Kit. Plasmid DNA concentrations were determined by measuring the absorbances at 260nm (A_{260}), and protein contamination was accessed by determining the A_{280}/A_{260} ratio.

Determination of effective concentrations of doxycycline

The effective concentrations of Dox may vary with different cell lines and with different lots of antibiotic. Therefore, it is important to titrate the optimal concentration for full activation of gene expression. For this, a Dox dose-response curve was performed using the U2-OS-Luc Tet-On Cell Line provided with the Tet-On advanced kit. This premade double-stable Tet-On Cell Line can exhibit over 500-fold induction of luciferase upon addition of Dox to the culture medium.

The U2-OS-Luc Tet-On cells were plated at density of $3x10^5$ cells in each well of a 6-well plate containing 3 ml of complete medium (DMEM, with 10% FBS Tet-Free, 100 µg/ml Hyg, 1% G418 and 1% PenStrep) plus varying amounts ($0.1x10^{-3}$, $1x10^{-2}$, 0.1, 1.0, 10, 100 and 1000 ng/ml) of Dox. The cells were allowed to grow at 37ºC in humid atmosphere with 5% CO_2 for 48 h, washed and lysed according to the protocol of the Luciferase Assay System (Promega). Briefly, 100 µl of lysis buffer were added to the adherent cells, and the cells were scrapped from the wells and transferred to microcentrifuge tubes. The lysates were centrifuged at 12000 x g for 2 min at 4ºC, the supernatants collected, transferred to wells of a 96-well opaque plate containing the Luciferase Assay Reagent and mixed. Luciferase activity was measured in a Microplate luminometer (Lmax™) programed to perform a 2 second (sec) measurement delay followed by a 10 sec measurement read for luciferase activity.

Chemical transfections

To perform the transfections, we decided to use the FuGENE® HD Transfection Reagent (Roche), a nonliposomal method involving the formation of a complex with DNA and the transport of the complex into the cell.

Optimization Transfection Reagent:DNA ratio and incubation time

To try to achieve maximum efficiency of transfection, we optimized the ratio of FuGENE® HD Transfection Reagent to DNA and the time of incubation of the FuGENE® HD Transfection Reagent with DNA.

In a first approach, we transfected the cells with the plasmid coding for luciferase (pTRE2hyg2-Myc-Luc) using FuGENE® HD Transfection Reagent:DNA ratios between 1.5:1 to 4:1. One day before the transfection assay, the cells

monolayers were washed, trypsinized and plated in wells of a 12-well plate at density of 1.5×10^5 cells per well in 1 ml of medium (DMEM with 10% FBS Tet-Free), and incubated 24 h in humid atmosphere with 5% CO_2, to achieve approximately 80% confluence at the time of transfection.

The plasmid DNA was diluted in DMEM at a final concentration of 0.02 µg/µl and added to the FuGENE® HD Transfection Reagent, as shown in Figure 6.

Transfection reagent:DNA (µl:µg)	Diluent (DMEM) (µl)	FuGENE® HD Transfection Reagent (µl)	DNA (µg)
1.5:1	50	1.5	1
2.0:1	50	2.0	1
2.5:1	50	2.5	1
3.0:1	50	3.0	1
3.5:1	50	3.5	1
4.0:1	50	4.0	1

Figure 6. Preparation of transfection complexes for a 12-well

The transfection reagent:DNA complexes were incubated for 10, 15, 30 and 45 min at RT before being added to the cells. After incubation for 24 h at 37ºC in humid atmosphere with 5% CO_2, the cells were washed, and then the medium with the transfection complex was changed to complete medium (DMEM, 10% FBS Tet-Free, 1% G418 and 1% PenStrep) with Dox (100 ng/ml) and the cells incubated for further 24 h. Transfection efficiency was determined by measuring the luciferase expression.

Transfection efficiency was determined by measuring the luciferase expression. To prepare the cell lysates, the cells were washed and lysed with lysis buffer (10 mM Tris-HCl pH 8.0, 0.5% Triton X-100, 150 mM NaCl, 10% glycerol; 20 µl per $1.0\text{x}10^6$ cells) and centrifuged at 16100 x g for 15 min at 4ºC. The supernatants were diluted 1:1000 in distilled H_2O. Twenty microliters of each supernatant dilution were transferred to wells of a 96-well opaque plate containing the Luciferase Assay Reagent and luciferase activity was measured as described before.

In a second approach, we transfected the cells with the plasmids coding for AIP56 (pTRE2hyg2-Myc-AIP56), AIP56Nmut (pTRE2hyg2-Myc-AIP56Nmut) and AIP56C (pTRE2hyg2-Myc-AIP56C) with FuGENE® HD Transfection Reagent:DNA ratios of 3.5:1, 4:1 and 5:2. The transfection was performed as described above. Transfection efficiencies were evaluated by IF, as described below.

Optimization of the transfection protocol

In an attempt to increase the efficiency of transfection, we transfected the cells using three different protocols:

(1) Standard transfection: a cell monolayer was washed, trypsinized and plated in wells of a 12-well plate with sterile cover slips, at density of $1.5x10^5$ cells per well in 1 ml of medium (DMEM with 10% FBS Tet-Free), incubated 24 h in humid atmosphere with 5% CO_2, to achieve approximately 80% confluence at the time of transfection. This protocol is the one recommended by the FuGENE® HD Transfection Reagent manufacture.

(2) Transfected of fresh trypsinized cells: a cell monolayer was washed, trypsinized and plated at density of $2x10^5$ cells per well, as in (1), incubated 1 h in humid atmosphere with 5% CO_2, and transfected.

(3) Transfection of fresh trypsinized cells in suspension: a cell monolayer was washed and trypsinized; $2x10^5$ cells were transferred to eppendorf tubes and collected by centrifugation at 800 g for 2 min at RT. Each cell pellet was resuspended in 25 µl of DMEM, transferred to a tube containing the transfection complex in 25 µl of DMEM and the suspension incubated at RT for 15 min, before being plated in wells of a 12-well plate with sterile cover slips. This method was developed by M. Zhang et al. [35].

To prepare transfection complexes, 1 µg of DNA was diluted in 50 (for protocols 1 and 2) or 25 (for protocol 3) µl DMEM and then added to 3.5 µl of FuGENE® HD Transfection Reagent. The transfection reagent:DNA complexes were incubated for 45 min at RT, before being used for transfections. Plates with transfected cells were incubated at 37ºC in humid atmosphere with 5% CO_2 for 24 h, washed, and then the medium with the transfection complexes replaced by complete medium with Dox. The cells were incubated at 37ºC in humid

atmosphere with 5% CO_2 for further 24 h. Transfection efficiencies were evaluated by IF, as described below.

Physical transfection

Electroporation with Nucleofector™ Kit R (Lonza)

Nucleofector™ method is based on two components, the Nucleofector™ Device that induces the optimized electrical parameters for electroporation, in aluminium cuvettes, and Nucleofector™ Kit, which contains a specific Nucleofector™ solution and a supplement. To prepare the transfection solution to be used in the Nucleofector™ method, 18 µl of supplement was mixed with 82 µl of Nucleofector™ solution in a microcentrifuge tube according to manufacturer's instructions.

Cells at sub-confluence were trypsinized and cell pellets collected by centrifugation at 200 x g for 10 min at RT. Then, each cell pellet, containing $6x10^5$ cells, was resuspended in 100 µl of the transfection solution. Two micrograms of plasmid DNA (pTRE2hyg2-Myc-AIP56mut, pTRE2hyg2-Myc-AIP56N, pTRE2hyg2-Myc-AIP56Nmut and pTRE2hyg2-Myc-AIP56C) were added to each tube and the cell suspension was transferred into a cuvette and submitted to electroporation (Nucleofector™ I Device, Nucleofector™ program I-013, for high expression level). The addgene plasmid 11347, (for constitutive expression of GFP-tagged non-muscle myosin heavy chain IIA), kindly provided by the Molecular Microbiology Group of IBMC, was used as a positive control. After electroporation, 500 µl of pre-warmed medium was added to the cell suspension. Duplicate aliquots of 300 µl of the resulting cell suspension were transferred to wells of a 12-well plate containing sterile coverslips and previously loaded with 700 µl of medium (DMEM with 10% FBS Tet-Free). The plates were incubated at 37ºC in humid atmosphere with 5% CO_2 for 24 h, washed, and then the medium with the transfection complex replaced by complete medium with Dox. The cells were incubated at 37ºC in humid atmosphere with 5% CO_2 for further 24 h. Transfection efficiencies were evaluated by IF, as described below.

Immunofluorescence

For IF, cells were washed and fixed with freshly prepared 4% paraformaldehyde (Merck) in phosphate buffered saline (PBS; Lonza), for 10 min. Fixed cells were washed and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were incubated for 30 min in blocking solution (10% FBS, 0.1% Tween 20, in PBS), followed by incubation with a mouse anti-c-Myc antibody (Santa Cruz Biotechnology) diluted in blocking solution (1:50), for 1 h at RT in a wet chamber, and then washed 3 times for 5 min in PBS, at RT. An Alexa Fluor® 488 goat anti-mouse antibody (Invitrogen, Molecular Probes), diluted in blocking solution (1:2000) was used as a secondary antibody together with the actin stain Alexa Fluor® 594 Phalloidin (Invitrogen, Molecular Probes) diluted in blocking solution (1:40). Incubation last for 1 h at RT in a wet chamber and in the dark. The slides were washed 3 times for 5 min in PBS at RT before being counterstained with 0.2 µg/ml of DAPI, in a wet chamber and in the dark. Slides were mounted with Vectashield® Mounting Media and analysed under a Zeiss AxioImager Z1 fluorescence microscope. Images were captured using an Axiocam MR Version 3.0 (Carl Zeiss, Germany). Cells from 10 randomly selected microscope fields were counted.

Stable transfection

Titration of hygromycin

Prior to use Hyg to establish stable cell lines, it is important to titrate the selection agent stocks to determine the optimal concentration for selection of resistant cells.

For titration of Hyg, the HeLa Tet-On cells were plated at density of $6x10^5$ cells in each of six 10 cm tissue culture dishes containing 10 ml of complete medium (DMEM, with 10% FBS Tet-Free, 1% G418 and 1% PenStrep) plus varying concentrations (0, 50, 100, 200, 400, 800 µg/ml) of Hyg. The cells were incubated for 14 days at 37ºC in humid atmosphere with 5% CO_2, replacing the selective medium every four days. The dishes were examined for the presence of viable cells every two days. At day 5, the plates were photographed under a Zeiss

Axiovert 200M inverted microscope using the 20 x objective. Cells from 6 randomly selected microscope fields were counted.

Transfection

To establish stable-transfected cell lines, freshly trypsinized HeLa Tet-On cells were transfected in suspension with FuGENE® HD Transfection Reagent, plated in wells of a 6-well plate and subjected to selection with Hyg.

To prepare transfection complexes, 2 µg of DNA was diluted in 50 µl of DMEM and then added to 7 µl of FuGENE® HD Transfection Reagent. The transfection reagent:DNA complexes were incubated for 45 min at RT, before being used for transfections.

A cell monolayer was washed and trypsinized; $6x10^5$ cells were transferred to eppendorf tubes and collected by centrifugation at 800 g for 2 min at RT. Each cell pellet was resuspended in 50 µl of DMEM, transferred to a tube containing the transfection complex in 50 µl of DMEM and the suspension incubated at RT for 15 min, before being plated in wells of a 6-well plate.

Plates with transfected cells were incubated at 37ºC in humid atmosphere with 5% CO_2 for 24 h, washed, and then the medium with the transfection complexes replaced by selective medium (DMEM, 10% FBS Tet-Free, 1% G418, 1% PenStrep and 50 µg/ml Hyg), and the plates were incubated again at 37ºC in humid atmosphere with 5% CO_2. The medium was replaced with fresh selective medium with Hyg every four days, until resistant cell colonies began to appear.

Intracellular delivery of AIP56 subunits using the LF-PA of system Bacillus anthracis

Cell culture

Raw 264.7 cells were maintained in DMEM with 10% FBS at 37ºC in humid atmosphere with 5% CO_2. Cells were sub-cultured whenever approximately 95% confluence was reached.

Delivery of AIP56 subunits

In this work, the following chimeric proteins were used: LFN, consisting of the anthrax protective antigen (PA) recognition domain of anthrax lethal factor (LF^{1-254}) fused with putative AIP56 A domain ($AIP56N^{1-261}$) and LFC, consisting of LF^{1-254} fused with putative AIP56 B domain ($AIP56C^{299-497}$). All the proteins were produced and are available at the Fish Immunology and Vaccinology group of IBMC.

One day before the assay, the cells were plated in a 96-well plate ($2x10^4$ cells/well). The fusion proteins (20 nM LFN or 20 nM LFC,) were added to the cells together with the 10 nM PA and cells were incubated for 6 h at 37ºC in humid atmosphere with 5% CO_2.

SDS-PAGE and western-blotting

To obtain cell lysates, cells were washed 3 times with PBS and lysed with GLB. DTT was added to the lysates at a final concentration of 100 mM, and the proteins denatured by heating at 95ºC for 5 min and resolved in 10% SDS-polyacrylamide gels, as described above.

Proteins in the gels were transferred to nitrocellulose membranes in transfer buffer, for 1 h at 21 V. To control transfer efficiency, equal loading of the lanes, and to locate the molecular weight markers, the membranes were stained with Ponceau S. The membranes were blocked for 1 h at RT in T-TBS and 5% low fat powdered milk and incubated for another hour at RT with shaking with rabbit anti-NF-kB p-65 antibody (C20;Santa Cruz Biotech), diluted in T-TBS (1:3000), washed 3 times for 5 min in T-TBS, at RT with shaking and incubated with HRP anti-rabbit IgG antibody (The Binding Site), diluted in T-TBS (1:30000), for 1 h at RT, with shaking. The membranes were washed again 3 times for 5 min in T-TBS, at RT with shaking, and the reactive bands were detected using ECL following the manufacturer's instructions.

Results and Discussion

In vitro cleavage of NF-kB p-65 by AIP56

In the beginning of this work there was a hypothesis that AIP56 was not internalized by mammalian cells, possibly due to lack of specific membrane receptors able to bind the toxin. That hypothesis came from the fact that when AIP56 was incubated with different mammalian cell lines, there were no signs of apoptosis and no toxin could be detected associated to the cells. Considering the homology between AIP56 N-terminal domain and NleC, a type-III secreted effector found in enteric bacteria pathogenic to mammals, it was further hypothesised that both bacterial proteins could have common targets/substrates. Therefore, when, during the course of this work, NF-kB p-65 was identified as a substrate of the NleC [17-20], we decided to investigate the ability of AIP56 to cleave the mammalian NF-kB p-65.

To elucidate the AIP56 proteolytic activity against NF-kB p-65, we carried out *in vitro* cleavage assays. HeLa Tet-On cell lysates were incubated with AIP56 or AIP56 related proteins and the NF-kB p-65 cleavage assessed by western-blotting (Figure 7).

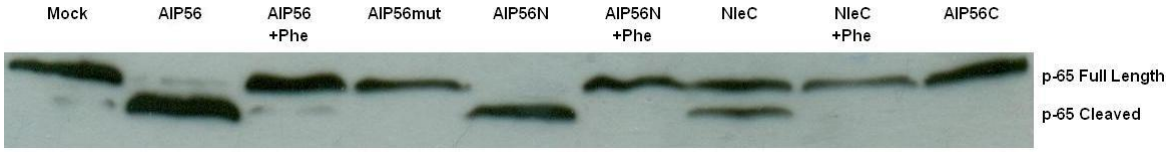


Figure 7. AIP56 cleaves mammalian NF-kB p-65 *in vitro*. Western blot of HeLa Tet-On cell lysates incubated with the indicated proteins for 3 h in the presence or absence of 5 mM of the metallopeptidase inhibitor 1,10-phenanthroline (Phe).

We found that AIP56, similarly to what has been described for NleC, cleaves NF-kB p-65 and that cleavage is inhibited by the metallopeptidase inhibitor 1,10- phenanthroline (Figure 7). Using the AIP56mut (AIP56 mutated in the metalloprotease signature), we also found that amino-acid substitutions in the zinc metalloprotease signature of the toxin abolished its proteolytic activity, confirming that cleavage of NF-kB p-65 by AIP56 is dependent on the metalloprotease activity of the toxin. In line with this conclusion are the results obtained with the AIP56 truncates, showing that the AIP56N (corresponding to the putative AIP56 A domain) retains the ability to cleave NF-kB p-65, whereas the AIP56C (corresponding to the putative AIP56 B domain) does not.

The identification of NF-kB p-65 as a substrate for AIP56 opens many prospects in what concerns the study of the AIP56's activities. Using NF-kB p-65 cleavage as an indication of AIP56 activity has already allowed the re-evaluation of the interaction of AIP56 with mammalian cell lines. Results obtained at the Fish Immunology and Vaccinology group of the IBMC show that, contrarily to what has been initially hypothesized, AIP56 is able to enter and cleave the NF-kB p-65 in several mammalian cell lines (HeLa, Caco-2, J774A.1, Raw 264.7), despite no apoptosis of those cells was observed after incubation with the toxin.

Intracellular delivery of AIP56-related proteins by transfection

Preliminary titrations

Prior to establish a transfection protocol with Tet-On cells, it is important to titrate the concentration of doxycycline (Dox) for optimal induction of gene expression as well as the concentration of hygromycin (Hyg) to be used in the selection of transfected cells when stable transfected cell lines are to be developed.

The Dox is used to induce the expression of the gene of interest. In order to determine the Dox concentration yielding the maximum expression of the required proteins, the expression of the luciferase in U2-OS-Luc cells was induced with different doses of Dox, and the luciferase expression quantified by luminescence at 48 h post-induction.

The highest luciferase activity was obtained with 100 ng/ml of Dox (Figure 8) and, therefore, this concentration was selected for further experiments.

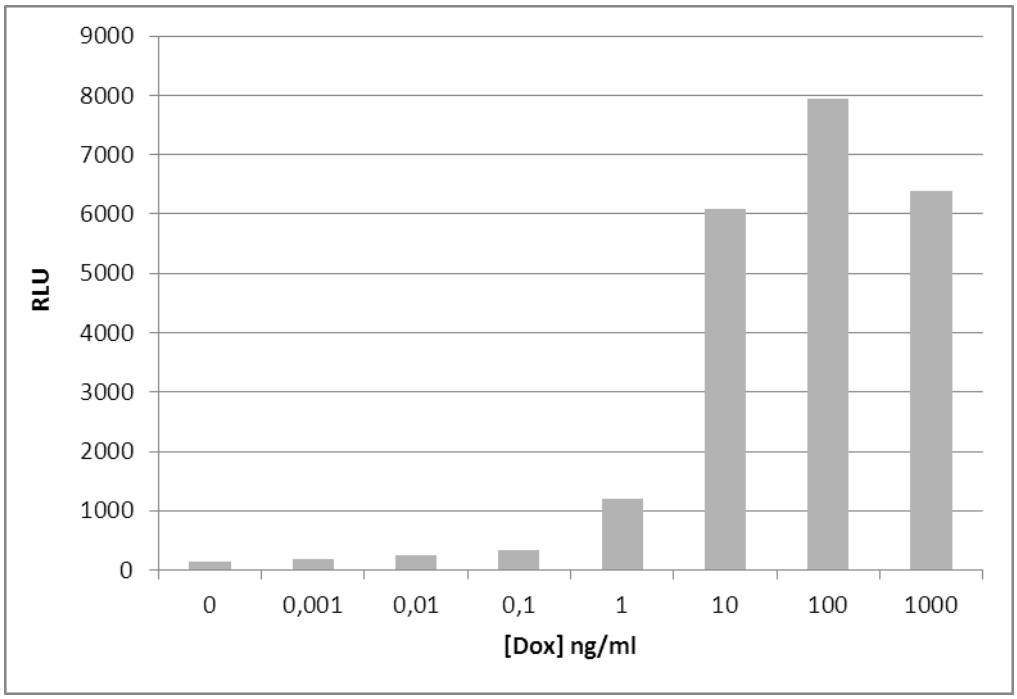


Figure 8. Doxycyclin dose-response assay. U2-OS-Luc Tet-On cells were plated at density of $3x10^5$ cells in each well of a 6-well plate in the presence of the indicated Dox concentrations. The cells were incubated 48 h, lysed, and the luciferase activity quantified by luminescence and expressed as relative luminescence units (RLU). Results shown are representative of 5 independent experiments

The Hyg is an antibiotic used to establish stable cell lines. The transfected cells have the plasmid that encodes for Hyg resistance. To choose the ideal concentration of Hyg, a dose response assay with different concentrations of antibiotic was performed. The optimal concentration is the lowest concentration that begins to induce massive cell dead, in non-transfected cells, at day 5 and kills all the cells within 14 days. These criteria gave the guidelines to choose the concentration that is enough to kill the cells without resistance (non-transfected) and not in excess to kill by toxicity the resistant transfected cells.

In Figure 9, the obtained kill curve is depicted, showing that 50 µg/ml Hyg induce approximately 50% cell dead at the 5^{th} day. When incubated with this concentration of Hyg, no viable cells were present at day 14^{th} (data not shown).

Considering these results a Hyg concentration of 50 µg/ml was chosen for subsequent experiments.

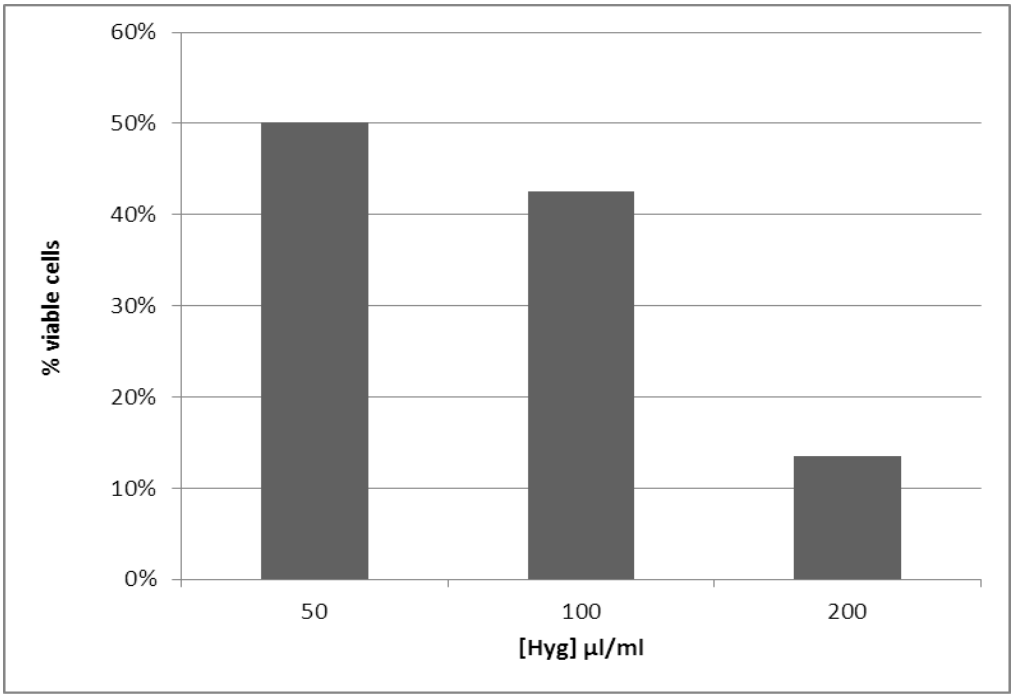


Figure 9. Hygromycin dose response assay. U2-OS-Luc Tet-On cells were plated at density of $6x10^5$ cells in 10 cm culture dishes with the indicated concentrations of Hyg and incubated up to 14 days. The results represent the viable cells counted at day 5, expressed in percentage relative to control cells (incubated in the absence of Hyg). Results shown are representative of 4 independent experiments.

Chemical transfection

Effect of transfection reagent:DNA ratio

In this work, chemical transfections were done using FuGENE® HD Transfection Reagent, a reagent that forms a complex with the plasmid DNA, and then transports the complex into cells. It is known that the transfection reagent:DNA ratio can influence the efficiency of transfection [36, 37]. To maximize the efficiency of transfection, optimization experiments, using different ratios of FuGENE® HD Transfection Reagent to DNA were performed. In a first approach, different ratios were compared using the plasmid coding for luciferase (pTRE2hyg2-Myc-Luc) for transfections and measuring the luciferase activity after 24 h of Dox induction.

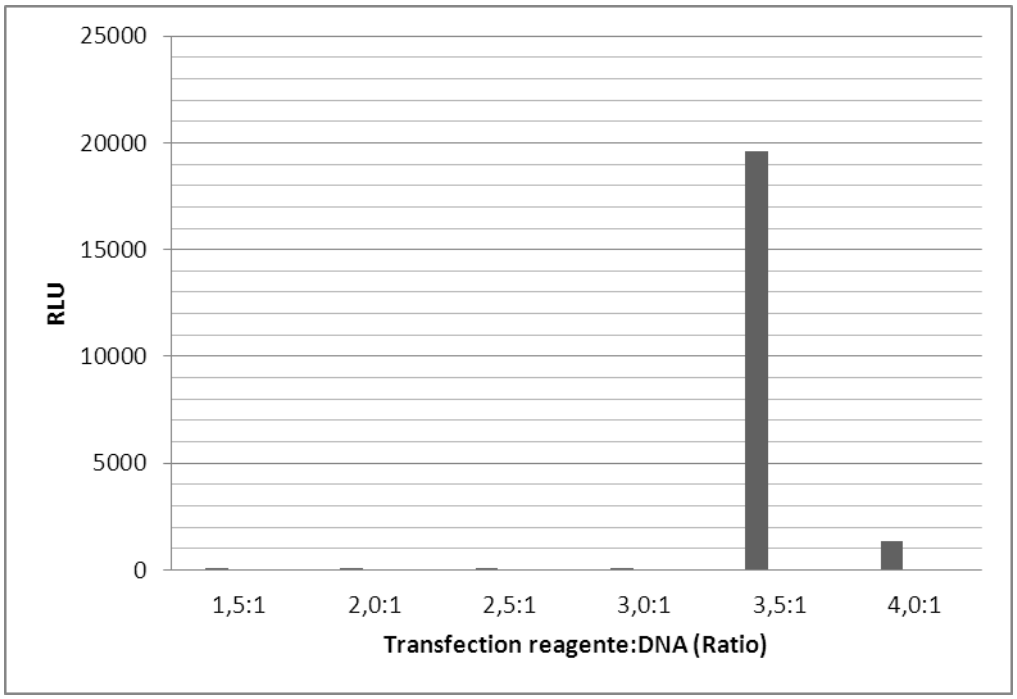


Figure 10. Optimization of the FuGene® HD Transfection Reagent:DNA ratio using pTRE2hyg2-Myc-Luc . Hela Tet-On cells were plated at density of 1.5×10^5 cells in each well of a 12-well plate with DMEM with 10% FBS Tet-Free and then transfected with the pTRE2hyg2-Myc-Luc by incubation with the indicated transfection Reagent:DNA ratios. After incubation for 24 h cells were induced with Dox (100 ng/ml), incubated for 24 h, lysed, and the luciferase activity quantified by luminescence and expressed as relative luminescence units (RLU).

The results show that the highest efficiency of transfection (resulting in the highest luciferase activity), was obtained with 3.5:1 transfection reagent:DNA ratio (Figure 10). In a second approach, the effect of the FuGENE® HD Transfection Reagent to DNA ratio was evaluated using plasmids pTRE2hyg2-Myc-AIP56, pTRE2hyg2-Myc-AIP56Nmut or pTRE2hyg2-Myc-AIP56C, using transfection reagent:DNA ratios of 3.5:1, 4.0:1 and 5.0:2. Regardless of the plasmid used, the highest percentage of transfected cells was obtained using the Transfection Reagent:DNA ratio of 3.5:1 (Figure 11), in accordance with the results obtained in the preliminary experiments with plasmid pTRE2hyg2-Myc-Luc (Figure 10).

Effect of incubation time of the transfection complexes

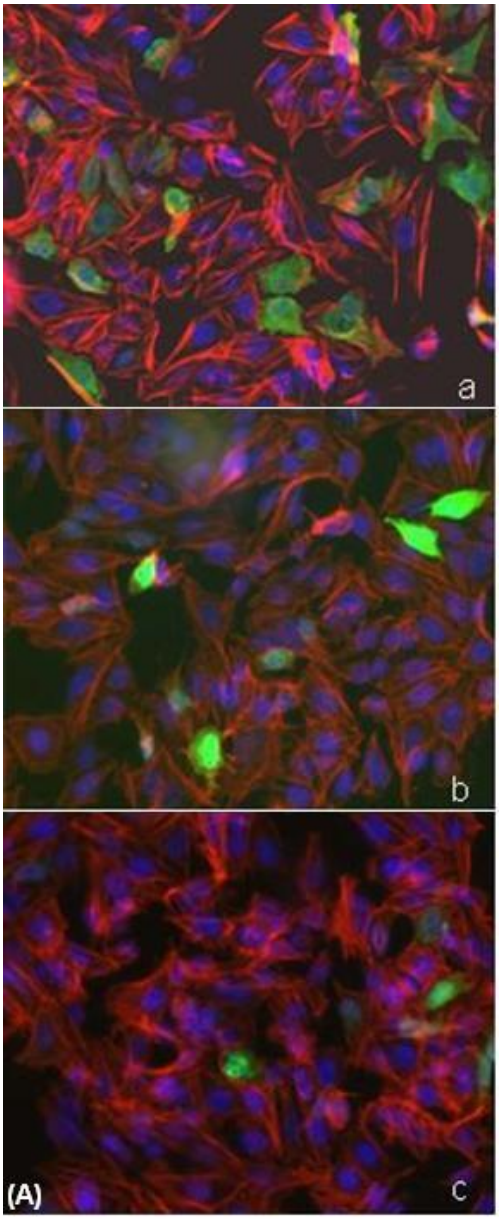
Because formation of the complexes involves interactions between the transfection reagent and the DNA [37], and also because the size of micelles has implication for the transfection [38], different incubation times (10, 15, 30, 45 min) of the transfection reagent:DNA complexes were tested, in order to determine the incubation time leading to the highest efficiency of transfection.

Immunofluorescence analysis of transfected cells showed that maximum efficiency of transfection was obtained when the transfection reagent:DNA complexes were incubated for 45 min, as compared to complexes incubated for 10, 15 and 30 min (data not shown).

Optimization of the transfection protocol

The transfection efficiency is crucial to obtain stable cell lines, because if the transfection has low efficiency, few cells will be selected to resist at the time of the selection with Hyg, therefore cells won't be enough to remain viable and form clones. It is usually considered that a suitable performance is 50% of transfected cells.

In the current work, when we performed the transfection using FuGENE® HD according to the manufacturer's instructions (protocol 1), regardless the transfection reagent:DNA ratio, transfection of HeLa Tet-On cells with pTRE2hyg2-Myc-AIP56, pTRE2hyg2-Myc-AIP56Nmut or pTRE2hyg2-Myc-AIP56C always resulted in less than 50% transfected cells, as assessed by positive IF staining (Figure 11). Regardless of the plasmid used, the highest percentage of transfected cells was obtained using the Transfection Reagent:DNA ratio of 3.5:1, and for this ratio, the highest percentage of transfected cells (36.57%) was obtained with plasmid pTRE2hyg2-Myc-AIP56C, while transfection with plasmids pTRE2hyg2-Myc-AIP56AIP56 and pTRE2hyg2-Myc-AIP56Nmut resulted in 21.71% and 17.84% transfected cells, respectively (Figure 11). The low efficiency of transfection can result from the fact that the receiver cells are killed due to injuries occurred during the transfection process or the complexes undergo lysosomal degradation [39, 40]. It can also be that the plasmids stay in the nucleus as an episomal DNA, without integrate into the host genome; these molecules in dividing cells do not replicate and are eventually diluted away as the population of replicating cells grows [39].



Transfection reagent:DNA (µl:µg)	Efficiency of transfection (%)		
	pTRE2hyg2-Myc-AIP56	pTRE2hyg2-Myc-AIP56Nmut	pTRE2hyg2-Myc-AIP56C
3.5:1	21.71	17.84	36.57
4.0:1	17.66	14.31	28.58
5.0:2	9.25	7.41	12.89

(B)

Figure 11. Effect of the FuGENE® HD Transfection Reagent:DNA ratios in the efficiency of transfection (A) Immunofluorescence analysis of the expression of AIP56Nmut in HeLa Tet-On cells transfected with pTRE2hyg2-Myc-AIP56Nmut using a (a) 3.5:1, (b) 4:1 or (c) 5:2 ratio of FuGENE® HD Transfection Reagent:DNA. After incubation for 24 h, transfected cells were induced with 100 ng/ml Dox for 24 h, fixed and processed for IF. The c-myc-tagged AIP56Nmut appears in green, nuclei were counterstained with DAPI (blue) and the actin cytoskeleton with Alexa Fluor® 594 Phalloidin (red). (B) Table summarizing the transfection efficiencies obtained for different plasmids and different FuGENE® HD Transfection Reagent:DNA ratios. Images obtained as in panel A served as the basis for quantifications.

The different efficiencies obtained with different plasmids may be related to the Zeta Potential, as described by S. Yamano et al. [41], because different plasmids interact with the lipids of the reagent and form complexes with different charges and these differences can affect the efficiency of the interaction of the complexes with the cell membrane.

Considering that the experiments performed with the FuGENE® HD Transfection Reagent using the standard protocol (protocol 1) always resulted in less than 50% transfected cells, we decided to optimize the transfection protocol in an attempt to obtain higher efficiencies of transfection. One of the approaches (protocol 2) consisted in doing the transfection essentially as described in the FuGENE® HD Transfection Reagent manual, but using freshly trypsinized cells (1 h after plating) instead of cells plated 24 h before. Using this method, the efficiencies increased slightly, but regardless of the plasmid used, did not reach the 50% transfected cells, as shown in Figure 12. Similarly to what was observed in transfections done using the standard protocol (protocol 1; Figure 11), the highest efficiency was obtained in cells transfected with plasmid pTRE2hyg2-Myc-AIP56C.

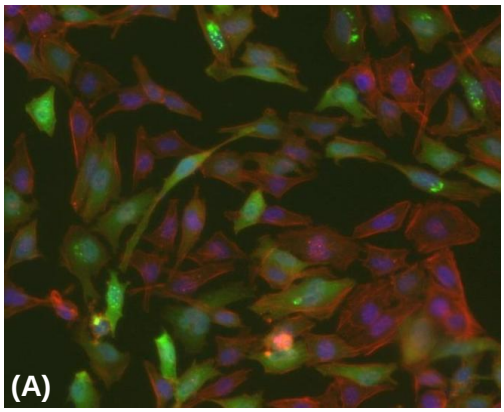
Transfected plasmid	Efficiency of transfection (%)
pTRE2hyg2-Myc-AIP56	24.77
pTRE2hyg2-Myc-AIP56Nmut	26.65
pTRE2hyg2-Myc-AIP56C	45.51

Figure 12. Percentages of transfected cells obtained by transfection of freshly trypsinized cells (1h after plating) with the indicated plasmids. Transfected cells were processed for IF and efficiencies of transfection determined as described in Figure 11.

Another approach tested in this work, consisted in transfecting freshly trypsinized cells in suspension with the FuGENE® HD Transfection Reagent (protocol 3). This protocol was developed by M. Zhang et al. [35], and, according to the authors, results in enhanced efficiencies of transfection.

When we used protocol 3 for transfection, regardless of the plasmid used, the percentage of transfected cells increased markedly for values always above

50% (Figure 13). The lowest efficiency of transfection (55.56%) was obtained using plasmid pTRE2hyg2-Myc-AIP56, while when plasmids pTRE2hyg2-Myc-AIP56Nmut and pTRE2hyg2-Myc-AIP56C were used, the efficiencies were 71.87% and 82.77%, respectively.



Transfected plasmid	Efficiency of transfection (%)
pTRE2hyg2-Myc-AIP56	55.56
pTRE2hyg2-Myc-AIP56Nmut	71.87
pTRE2hyg2-Myc-AIP56C	82.77

(B)

Figure 13. Percentages of transfected cells obtained by transfection of freshly trypsinized cells in suspension with the indicated plasmids. (A) Immunofluorescence analysis of expression of AIP56Nmut in HeLa Tet-On cells transfected with pTRE2hyg2-Myc-AIP56Nmut using a 3.5:1 ratio of FuGENE® HD Transfection reagent:DNA. After incubation for 24 h, transfected cells were induced with 100 ng/ml Dox for 24 h, fixed and processed for IF. The c-myc tagged AIP56Nmut appears in green, nuclei were counterstained with DAPI (blue) and the actin cytoskeleton with Alexa Fluor® 594 Phalloidin (red). (B) Table summarizing the transfection efficiencies obtained for different plasmids. Images obtained as in panel A served as the basis for quantifications.

Physical transfection

Exposing eukaryotic cells to brief, high voltage electrical fields can induce the uptake of exogenous materials, presumably through the transient formation of micropores in the cell membrane [42]. When cells are transfected by electroporation, such electrical field is used to alter the cell permeability producing transient holes in the cell membrane, through which the DNA can enter [43].

In this work, we transfected HeLa Tet-On cells by electroporation with plasmids pTRE2hyg2-Myc-AIP56mut, pTRE2hyg2-Myc-AIP56N, pTRE2hyg2-Myc-AIP56Nmut, pTRE2hyg2-Myc-AIP56C using the Nucleofector™ Kit R. Regardless

of the plasmid used for electroporation, few cells were recovered after the process and the ones that remained viable were not expressing the myc-tagged proteins, as concluded from negative IF staining (Figure 14). This could be due to injuries caused to cells during the transfection, because although electroporation is considered an efficient method to transfect almost all types of cells with some success, it is, at the same time, a severe technique and many cells die in the process [39, 44].

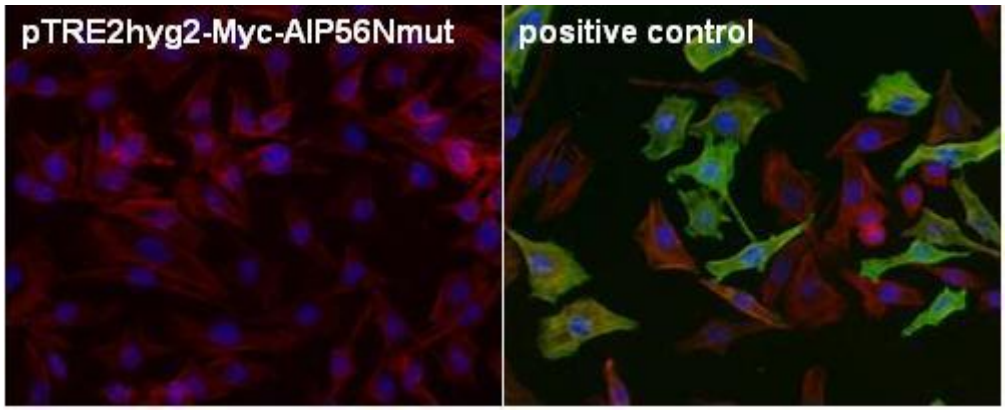


Figure 14.Immunofluorescence analysis of expression of AIP56Nmut in HeLa Tet-On cells transfected by electroporation with pTRE2hyg2-Myc-AIP56Nmut (left panel). After transfection, cells were plated, incubated for 24 h, induced with 100 ng/ml Dox for 24 h, fixed and processed for IF. Cells transfected with a plasmid coding for GFP-tagged NMHC II-A were used as a positive control **(right panel).** The c-myc tagged AIP56Nmut or GFP-tagged NMHC II-A appear in green, nuclei were counterstained with DAPI (blue) and the actin cytoskeleton with Alexa Fluor® 594 Phalloidin (red).

Stable transfection

As already discussed, the success of the establishment of stable-transfected cells lines is much dependent in the initial number/percentage of transfected cells at the time of the selection with the selective agent. The higher the number of transfected cells, the higher the probability of success in obtaining stable-transfected clones after selection. In an attempt to obtain stable-transfected cell lines, we started by using the standard transfection protocol recommended by the FuGENE® HD Transfection Reagent manufacturer (protocol 1) followed by selection with Hyg, according to the results obtained in the preliminary titration assays. However, although some transfected cells remained viable for more than two weeks, they did not proliferate to form clones (not shown). This was probably because the initial number of transfected cells was too low to allow them to proliferate. Therefore, after the above-discussed optimizations of the transfection protocol that allowed us to greatly increase the transfection efficiencies, we set up

new experiments in which we transfected freshly trypsinized cells in suspension with the FuGENE® HD Transfection Reagent and proceeded to selection with Hyg. These experiments are currently ongoing and we expect to obtain stable-transfected clones in the near future.

Intracellular delivery of AIP56 subunits using the LF-PA system of Bacillus anthracis

Anthrax toxin is composed of three separate proteins produced by *Bacillus anthracis*: lethal factor (LF), edema factor (EF), and protective antigen (PA). This toxin belongs to the family of bacterial binary AB-toxins and is capable of binding and entering almost any cell type [45]. PA binds to a cellular receptor, the anthrax toxin receptor (ATR), and mediates the delivery of the enzymatic components (LF and EF) to the cytosol (Figure 15). PA is cleaved by a furin-like cell-surface membrane protease, releasing the PA_{20} subunit in the extracellular milieu. The remaining PA_{63} subunit oligomerizes to form $[PA_{63}]_7$ that binds a maximum of three molecules of LF [46].

The assembled toxins are endocytosed and tracked to endosomes that are moved to a low-pH endosomal compartment where the acidic environment induces a conformational change in PA that allows it to insert into the endosome membrane and form a pore, promoting the translocation of LF to the cytosol to cause death of macrophages [45].

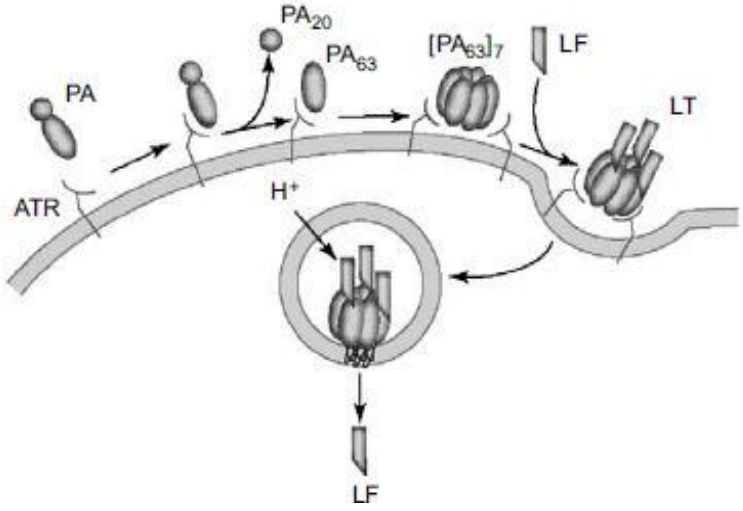


Figure 15. Schematic model of Anthrax lethal toxin translocation. Adapted from [46]

Using fusion proteins containing a portion of LF and the catalytic domains of some toxins, it has been demonstrated that amino acid residues 1 to 254 of LF (LF^{1-254}) are the minimum region required to recognize and bind the protective antigen (PA) and to be sufficient to cause cellular uptake of fused polypeptides [47] such as the catalytic domains of diphtheria, shiga and pseudomonas toxins to cell cytosol [47-50]. Most cell types possess receptors to the PA, and the possibility to construct fusions between the non-catalytic domain of the LF (LF^{1-254}) and other proteins, opens the possibility to use the LF-PA system as a vehicle to delivery proteins into the cytosol of cells [49-51].

In this work we used LFAIP56 chymeric proteins (LFN and LFC) together with PA to access the ability of the LF-PA system to deliver the AIP56 catalytic domain into the cytosol of Raw 264.7 cells. Figure 16 shows that this system was able to efficiently deliver the AIP56 catalytic domain, as confirmed by the NF-kB p-65 cleavage in cells incubated with PA+LFN. PA+LFC was used as a negative control and, as expected, when incubated with cells did not result in NF-kB p-65 cleavage.

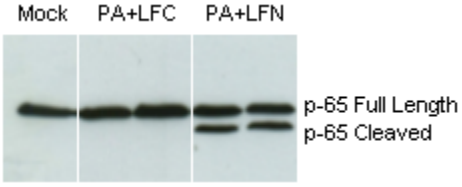


Figure 16. Western blotting analysis of NF-kB p-65 cleavage after incubation of Raw 264.7 cells with LFN or LFC in the presence of PA for 6 h.

Conclusion

During the course of this work, the understanding of the NleC, the N-terminal homologue of AIP56 has advanced dramatically and NF-kB p-65 was reported as NleC's first identified substrate. The homology between NleC and AIP56 led us to investigate the ability of AIP56 to cleave NF-kB p-65. We found that, similarly to NleC, AIP56 cleaves NF-kB p-65, although the relationship between the p-65 cleavage and the apoptotic activity of AIP56 remains to be elucidated. The identification of NF-kB p-65 as a substrate for AIP56 has already allowed the re-evaluation of the interaction of AIP56 with mammalian cell lines showing that, contrarily to what has been initially hypothesized, AIP56 is able to enter and cleave the NF-kB p-65 in several mammalian cell lines.

The lack of commercially available tools to study the activity of AIP56 in sea bass cells prompted us to develop a mammalian cell model to study the activity of the toxin. Different protocols were tested and optimized for the intracellular delivery of AIP56 or AIP56-related proteins into mammalian cells and, from the results obtained, we can conclude that the most efficient methods for the delivery of the proteins were: (1) chemical transfection of HeLa Tet-On cells with toxin-encoding vectors using FuGENE® HD Transfection Reagent by an alternative method for efficiency enhancement, in which freshly trypsinized cells are transfected in suspension (in contrast to the standard protocol, in which cells are transfected in monolayers 24 h after plating); (2) the use of the LF-PA system of *Bacillus anthracis*. The chemical transfection (either transient or stable) of HeLa Tet-On cells may be very advantageous whenever inducible expression of high levels of the AIP56 or AIP56-related proteins is necessary, because it allows the controlled induction of protein synthesis. In contrast, the LF-PA system of *Bacillus anthracis* may be more suitable when delivery of low amounts of AIP56 or AIP56-related proteins through a more physiological route is preferable.

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